Table 4. NCI–CTC grade 3/4 toxicities ($n = 40$, recommended regimen)

Toxicities	Grade 3		Grade 4	
	n	%	*n*	%
Hematological toxicities				
Leukopenia	13	32.5	2	5.0
Neutropenia	12	30.0	11	27.5
Lymphopenia	5	12.5	0	0.0
Hemoglobin decreased	2	5.0	0	0.0
Thrombocytopenia	1	2.5	0	0.0
Thrombocytosis	1	2.5	0	0.0
Non-hematological toxicities				
ALT increased	2	5.0	0	0.0
Diarrhea	2	5.0	0	0.0
Infection	2	5.0	0	0.0
Interstitial pneumonia	2	5.0	0	0.0
Rash	2	5.0	0	0.0
Fatigue	2	5.0	0	0.0
Nausea	2	5.0	0	0.0
Vomiting	2	5.0	0	0.0
Hyperglycemia	1	2.5	0	0.0
Hyponatremia	1	2.5	0	0.0
AST increased	1	2.5	0	0.0
Allergic reaction	1	2.5	0	0.0
Vasovagal reaction	1	2.5	0	0.0
Anorexia	1	2.5	0	0.0
Body temperature decrease	1	2.5	0	0.0
Weight increase	1	2.5	0	0.0
Hypotension	1	2.5	0	0.0
Pneumonia	1	2.5	0	0.0
Edema	1	2.5	0	0.0
Constipation	1	2.5	0	0.0
Peripheral neuropathy	1	2.5	0	0.0
Anaphylaxis	0	0.0	2	5.0

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

well-examined regimens. In recent studies using gemcitabine–docetaxel in NSCLC, response rates of 25–50% (19,25–29) and time-to-progression of disease of 106–132 days (31,32) have been reported. Georgoulias et al. (16) reported that the gemcitabine–docetaxel and docetaxel–cisplatin regimens they compared were equivalent in efficacy, but toxicity was severe in the latter. While docetaxel–cisplatin regimens showed severe toxicities of grade 3 anemia (5%), grade 3/4 neutropenia (13%/21%), grade 3 nausea/vomiting (10%) and grade 3 diarrhea (8%), gemcitabine–docetaxel regimens had grade 3/4 anemia (1%/1%), grade 3/4 neutropenia (11%/11%), grade 3 nausea/vomiting (2%) and grade 3/4 diarrhea (2%/1%) in 441 patients. However, the difference of efficacy and safety by the administration schedule and dosage of gemcitabine and docetaxel has not been well documented.

There are some studies that have examined the efficacy and safety of the same schedule as the recommended regimen in our study, namely gemcitabine on days 1 and 8 plus docetaxel on day 1. In these studies dosages were various: gemcitabine was 800–1100 mg/m^2 and docetaxel was 60–100 mg/m^2 (18,19,27–30). Response rates in these studies also varied from 16 to 38%, which indicates that the response rate of the recommended regimen in our study (30.0%) was clinically meaningful because the dosage of docetaxel (50 mg/m^2) in our study is less than that in any other studies. This might have contributed to the relatively mild toxicities of our recommended regimen.

In another study (26), a high response rate (50.0%) was achieved in patients with another administering schedule: gemcitabine 1000 mg/m^2 on days 1 and 10 plus docetaxel 80 mg/m^2 on day 1, administered every 21 days. The most common treatment-related toxicity was myelosuppression. Grade 3/4 leukopoenia and neutropenia occurred in only six (18%) and eight (24%) patients, respectively.

The median survival was 11.9 months in our study, being slightly better than the result from the median survival of the phase III study with gemcitabine and cisplatin, which was 8.7–9.1 months (33,34). This result suggests that the regimen we selected in the phase II portion of this study is comparable in survival with the cisplatin-based regimen.

In conclusion, the combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be better tolerated and has equivalent efficacy to cisplatin-based therapy. These results should be verified by a phase III study in Japanese patients.

CONCLUSION

In this phase I/II study, we studied the activity and tolerability of gemcitabine and docetaxel in Japanese patients. The combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be well tolerated and has equivalent efficacy to cisplatin-based therapy.

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Phase I–II study of amrubicin and cisplatin in previously untreated patients with extensive-stage small-cell lung cancer

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Background: Amrubicin, a totally synthetic 9-amino-anthracycline, demonstrated excellent single-agent activity for extensive-stage small-cell lung cancer (ED-SCLC). The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy with amrubicin and cisplatin, and to assess the efficacy and safety at their recommended doses (RD).

Patients and methods: Eligibility criteria were patients having histologically or cytologically proven measurable ED-SCLC, no previous systemic therapy, an Eastern Cooperative Oncology Group performance status of 0–2 and adequate organ function. Amrubicin was administered on days 1–3 and cisplatin on day 1, every 3 weeks.

Results: Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2/day and cisplatin 60 mg/m^2) and three patients at level 2 (amrubicin 45 mg/m^2/day and cisplatin 60 mg/m^2). Consequently, the MTD and RD were determined to be at level 2 and level 1, respectively. The response rate at the RD was 87.8% (36/41). The median survival time (MST) was 13.6 months and the 1-year survival rate was 56.1%. Grade 3/4 neutropenia and leukopenia occurred in 95.1% and 65.9% of patients, respectively.

Conclusions: The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC.

Key words: anthracycline, cisplatin, phase I–II, small-cell lung cancer

Introduction

Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors, and the outcome of SCLC patients is slowly but surely improving. Combination chemotherapy consisting of cisplatin plus etoposide and concurrent twice-daily thoracic radiotherapy has yielded a 26% 5-year survival rate in limited-stage (LD) patients [1]. Despite the high response rate to combination chemotherapy, however, local and distant failure is very common, especially in extensive-stage (ED) patients. Moreover, resistance to chemotherapeutic agents develops easily after failure of initial treatment. Thus, long-term survivors are still very rare among patients with ED-SCLC. To improve the outcome of SCLC patients, several strategies, such as high-dose chemotherapy, dose-intensive chemotherapy, alternating chemotherapy and introduction of new drugs, have been investigated [2–6]. However, only the introduction of new agents has improved the outcome of SCLC patients. Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin alternating cyclophosphamide, doxorubicin and vincristine had been mainly used for SCLC in North America. Recently, a Japanese trial [Japan Clinical Oncology Group (JCOG) 9511] demonstrated the superiority of the combination of irinotecan and cisplatin for ED-SCLC patients over the combination of etoposide and cisplatin [6]. The development of more active chemotherapy, and especially the introduction of effective new drugs, is therefore essential to improve the survival of SCLC patients.

Amrubicin (SM-5887) is a totally synthetic anthracycline and a potent topoisomerase II inhibitor [7–14]. It has antitumor activity, and is more potent than doxorubicin against various mouse experimental tumors and human tumor

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xenografts. Amrubicin and its 13-hydroxy metabolite, amrubicinol, inhibit purified human DNA topoisomerase II [11]. Amrubicinol is 10–100 times more cytotoxic than amrubicin [9]. The potent therapeutic activity of amrubicin is caused by the selective distribution of its highly active metabolite, amrubicinol, in tumors [9]. In an experimental animal model, amrubicin did not exhibit any chronic cardiotoxicity potential, and no deleterious effects on doxorubicin-induced cardiotoxicity in dogs was observed [14]. In a phase II study of amrubicin using a schedule of 45 mg/m^2 on days 1–3 every 3 weeks, in 33 previously untreated ED-SCLC patients, an overall response rate of 76% and a complete response (CR) rate of 9% were reported [15]. Moreover, median survival time (MST) was 11.7 months in the single-agent phase II study of amrubicin. Amrubicin is one of the most active new agents for SCLC. Thus, we conducted a phase I/II study of amrubicin plus cisplatin for untreated ED-SCLC, because cisplatin is considered as one of the most important drugs in the treatment of SCLC. The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy of amrubicin with cisplatin, to assess the efficacy and safety for ED-SCLC at their recommended doses (RD), and to examine the pharmacokinetics of the drug combination.

Patients and methods

Patient selection

Patients with histologically and/or cytologically documented SCLC were eligible for this study. Each patient was required to meet the following criteria: extensive-stage disease [16]; no prior therapy for primary lesion; measurable lesion; Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–2; expected survival time >2 months; age 20–74 years; adequate hematological function [white blood cell (WBC) count 4000–12 000/mm^3, neutrophils ≥2000/mm^3, platelets ≥100 000/mm^3, hemoglobin ≥10 g/dl]; adequate hepatic function [total bilirubin within 1.5× the upper limit of normal; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5× the upper limit of normal]; adequate renal function (creatinine within the upper limit of normal); partial pressure of arterial oxygen 60 torr; no abnormality requiring treatment on electrocardiogram; left ventricle ejection fraction >60%; written informed consent. Patients with symptomatic brain metastasis, pleural effusion that required drainage, non-steroidal anti-inflammatory drug or glucocorticoid use for >50 days, pericarditis carcinomatous, active infection, varicella, superior vena cava syndrome, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), gastric and/or duodenal ulcer, severe heart disease, severe renal disease, active concomitant malignancy, symptomatic pneumonitis and/or pulmonary fibrosis and pregnant/nursing women were excluded. This study was approved by the Institutional Review Board at each hospital.

Patient evaluation

Pretreatment evaluation consisted of complete blood cell counts, differential, routine chemistry measurements, progastrin-releasing peptide (ProGRP), neuron-specific enolase, electrocardiogram, echocardiography, chest radiograph, chest and abdominal computed tomography (CT) scan, whole-brain magnetic resonance imaging (MRI) or CT scan, and isotope bone scan. Complete blood cell counts, differential and routine chemistry measurements were performed at least once a week during the chemotherapy.

Treatment schedule

At level 1, chemotherapy consisted of cisplatin 60 mg/m^2 on day 1 and amrubicin 40 mg/m^2 on days 1–3. Amrubicin was administered as an intravenous injection over 5 min and cisplatin was administered as a drip infusion over 60–120 min with adequate hydration. At level 2 the dose of amrubicin was increased to 45 mg/m^2 on days 1–3. Level 3 was planned with cisplatin 80 mg/m^2 on day 1 and amrubicin 45 mg/m^2 on days 1–3. The chemotherapy was repeated every 3 weeks for four to six courses. Intrapatient dose escalation was not allowed. Administration of granulocyte colony-stimulating factor (G-CSF) was permitted prophylactically for patients expected to experience grade 3 neutropenia during the first course. Prophylactic administration of G-CSF was only permitted a second or later courses.

The administrations of both cisplatin and amrubicin were postponed if patients met the following criteria: WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; AST and ALT >5× the upper limit of normal; total bilirubin >1.5× the upper limit of normal; creatinine >1.3× the upper limit of normal; ECOG PS 3 or 4; active infection; grade 2 or worse non-hematological toxicity, except for alopecia, anorexia, nausea, vomiting or fatigue.

The administrations of both cisplatin and amrubicin were withdrawn if patients met the following criteria: tumor regression <15% after first course or <30% after second course; WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; no recovery from grade 3 or 4 non-hematological toxicity at 6 weeks after the start of previous chemotherapy; abnormality of electrocardiogram requiring treatment for more than 6 weeks; left ventricle ejection fraction <48%; treatment delay of >4 weeks.

The dose of amrubicin was decreased 5 mg/m^2/day if patients met the following criteria: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 neutropenia with fever; platelets <20 000/mm^3 during the previous course. The dose of cisplatin was decreased to 75% if creatinine increased to >1.5× the upper limit of normal during the previous course.

The dose-limiting toxicity (DLT) was defined as follows: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 febrile neutropenia; platelets <20 000/mm^3; grade 3 or worse non-hematological toxicity except for nausea, vomiting, anorexia, fatigue, hyponatremia and infection. Initially, three patients were treated at each dose level. If DLT was not observed in any of the three patients, dose escalation was carried out. If DLT was observed in one of three patients, an additional three patients were entered at the same dose level. If DLT was observed in three or more of six patients, or two or three of the initial three patients, we considered that dose to be the MTD. If DLT was observed in one or two of six patients, dose escalation was also carried out. Dose escalation was determined based only on the data from the first course of chemotherapy.

Response and toxicity evaluation

Response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) and tumor markers were excluded from the criteria [17]. CR was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks and no new lesions. Partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameters of target lesion, taking as reference the baseline sum longest diameter, the required non-progression in non-target lesions and no new lesions for at least 4 weeks. Stable disease (SD) included: regression of target lesions insufficient to meet the criteria for PR, a <20% increase in the sum of the longest diameter of target lesion, taking as reference the smallest sum longest diameters recorded since the treatment started, the required non-progression in non-target lesions and no new lesions for at least 6 weeks. Progressive disease (PD) indicated a >20% increase in the sum of the longest diameters of target lesion, taking as reference the smallest sum longest diameter recorded since the treatment started

and/or unequivocal progression of existing non-target lesions and/or appearance of new lesions. The evaluation of objective tumor response for all patients was performed by an external review committee.

Toxicity grading criteria of the National Cancer Institute Common Toxicity Criteria (version 2.0) was used for evaluation of toxicity.

Statistical analysis

This study was designed to reject response rates of 70% (P0) at a significance level of 0.05 (one-tailed) with a statistical power of 80% to assess the activity of the regimen as a 85% response rate (P1) at the recommended dose. The upper limit of rejection was 29 responses (CR + PR) among 37 evaluable patients. Overall survival was defined as the interval between the first administration of the drugs in this study and death or the last follow-up visit. Median overall survival was estimated using the Kaplan–Meier method [18].

Table 1. Characteristics of treated patients

	Phase I	Phase II	Total
Number of patients	7	37	44
Gender			
Male	5	31	36
Female	2	6	8
Age (years)			
Median	65	64	64.5
Range	54–73	50–74	50–74
ECOG PS			
0	0	5	5
1	7	32	39
2	0	0	0
Stage			
IIIB	0	2	2
IV	7	35	42
Prior therapy			
Yes	0	1	1
No	7	36	43
Serum ALP			
Normal	7	29	36
Elevated	0	7	7
Serum LDH			
Normal	3	14	17
Elevated	4	23	27
Na			
Normal	6	35	41
Decreased	1	2	3
Number of metastases			
0	0	2	2
1	4	27	31
2	3	6	9
3	0	1	1
4 or more	0	1	1

In one patient, serum ALP level could not be measured.
ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

Pharmacokinetic analysis

Pharmocokinetic analysis was performed in patients entering the phase I section of this study. One milliliter of the blood was taken from the patients before administration of amrubicin, and at 0 min, 15 min, 1, 2, 3, 4, 8 and 24 h after administration on days 1 and 3 in the first course of chemotherapy. Concentrations of amrubicin and its active metabolite, amrubicinol, in plasma and red blood cells were measured as reported elsewhere [9].

Results

Patient characteristics

Between April 2001 and December 2002, 45 patients with ED-SCLC were enrolled and 44 were treated in this study (Table 1). One patient did not receive the protocol treatment because atrial fibrillation was observed just before administration on day 1 of the first course. All treated patients were assessed for response, survival and toxicity. The median age of the treated patients was 64.5 years (range 50–74). There were 36 males and eight females. Five patients had an ECOG PS 0 and 39 patients had PS 1. Only one patient received surgery for brain metastasis as a prior therapy.

MTD and DLT in the phase I study

Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1) and three patients at level 2 (amrubicin 45 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1). Toxicities in the phase I study are listed in Table 2. No DLT were observed during the first course of level 1. At level 2, grade 4 neutropenia for $\geq$4 days and febrile neutropenia occurred in one patient, and febrile neutropenia and grade 3 constipation occurred in another patient. Consequently, the MTD and RD were determined to be level 2 and level 1, respectively.

Pharmacokinetics of amrubicin and its active metabolite, amrubicinol

Pharmacokinetic parameters of amrubicin in plasma were almost identical on days 1 and 3 at the two dose levels (Table 3). No clear dose relationship in the area under the concentration–time curve (AUC) of amrubicin in the plasma was observed. The AUC of amrubicinol in red blood cells tended to increase on day 3 at both doses (Table 4). No clear dose relationship in the AUC of amrubicinol in red blood cells was observed. Combination with cisplatin did not alter the pharmacokinetics of amrubicin and amrubicinol (data not shown).

Treatment received in patients treated at the RD

Forty-one patients were treated at the RD: amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1. Of 41 patients, 32 (78%) patients received more than three

Table 2. Toxicities during the first course in the phase I study

	Level 1 ($n=4$)					Level 2 ($n=3$)				
Amrubicin	40 mg/m^2 days 1–3					45 mg/m^2 days 1–3				
Cisplatin	60 mg/m^2 day 1					60 mg/m^2 day 1				
	Grade (NCI CTC)					Grade (NCI CTC)				
	0	1	2	3	4	0	1	2	3	4
Leukopenia	0	1	1	2	0	0	0	1	1	1
Neutropenia	0	0	0	2	2	0	0	0	0	3
Febrile neutropenia	4	–	–	0	0	1	–	–	2	0
Hemoglobin	1	1	2	0	0	2	1	0	0	0
Thrombocytopenia	1	2	0	1	0	0	2	0	1	0
Stomatitis	3	0	1	0	0	3	0	0	0	0
Nausea	1	1	2	0	–	1	1	0	1	–
Constipation	3	0	1	0	0	1	0	1	1	0
Hyponatremia	2	1	0	0	1	1	2	0	0	0
Hypocalcemia	3	0	1	0	0	3	0	0	0	0

Dose limiting toxicity at level 2: febrile neutropenia, two patients; grade 4 neutropenia ≥4 days, one patient; grade 3 constipation, one patient.
NCI CTC, National Cancer Institute Common Toxicity Criteria.

Table 3. Pharmacokinetics of amrubicin in plasma

Dose	n	Day	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	V_d (l)	CL (l/h)	$AUC_{0-24\,h}$ (ng h/ml)
40 mg/m^2	4	1	0.11±0.04	2.29±0.31	46.6±11.0	13.6±1.8	2995±434
	4	3	0.08±0.01	2.89±0.34	50.0±10.6	11.6±1.9	3511±514
45 mg/m^2	3	1	0.13±0.05	2.39±0.34	56.3±10.6	14.9±1.8	3052±402
	3	3	0.09±0.03	2.27±0.18	51.9±3.7	14.2±2.3	3217±479

$T_{1/2\alpha}$, half-life at distribution phase; $T_{1/2\beta}$, half-life at elimination phase; V_d, volume of distribution; CL, clearance; AUC, area under the concentration–time curve.

courses of chemotherapy, and 10 (31%) of these 32 patients needed dose reduction of amrubicin at the fourth course (Table 5). Of 41 patients, 22 (54%) patients completed four courses of chemotherapy without dose modification. The main cause of dose reduction was myelosuppression, especially leukopenia and neutropenia.

Objective tumor response and overall survival

The objective tumor responses are given in Table 6. Four CRs and 32 PRs occurred, for an objective response rate of 87.8% [95% confidence interval (CI) 73.8% to 95.9%] in 41 patients treated at the RD. The objective response rate for all 44 patients was 88.6% (95% CI 75.4% to 96.2%). The overall survival times of the 41 patients treated at the RD are shown in Figure 1. The MST of the 41 patients was 13.6 months (95% CI 11.1–16.6), with a median follow-up time for eight censored patients of 16.4 months (95% CI 14.2–18.8). The 1- and 2-year survival rates were 56.1% and 17.6%, respectively. The MST of all 44 patients was 13.8 months (95% CI 11.1–16.6). The 1- and 2-year survival rates of all 44 patients were 56.8% and 21.4%, respectively.

Table 4. Pharmacokinetics of amrubicinol in red blood cells

Dose	n	Day	$T_{1/2}$ (h)	$AUC_{0-24\,h}$ (ng·h/ml)
40 mg/m^2	4	1	21.0±3.1	1412±314
	4	3	20.7±4.8	2159±622
45 mg/m^2	3	1	19.6±6.1	1098±277
	3	3	18.1±5.7	2027±332

$T_{1/2}$, elimination half-life; AUC, area under the concentration–time curve.

Table 5. Treatment received in patients treated at the recommended dose

Cycle	n	Amrubicin (mg/m^2)			Cisplatin (mg/m^2)	
		40	35	30	60	45
1	41	41			41	
2	36	30	6		36	
3	33	26	5	2	33	
4	32	22	8	2	32	
5	18	9	5	4	18	
6	13	6	3	4	12	1

Table 6. Response rates

	n	CR	PR	SD	PD	NE	Response rate (%) (95% CI)
All	44	4	35	3	0	2	88.6 (75.4–96.2)
Treated at RD	41	4	32	3	0	2	87.8 (73.8–95.9)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated; 95% CI, 95% confidence interval; RD, recommended dose.

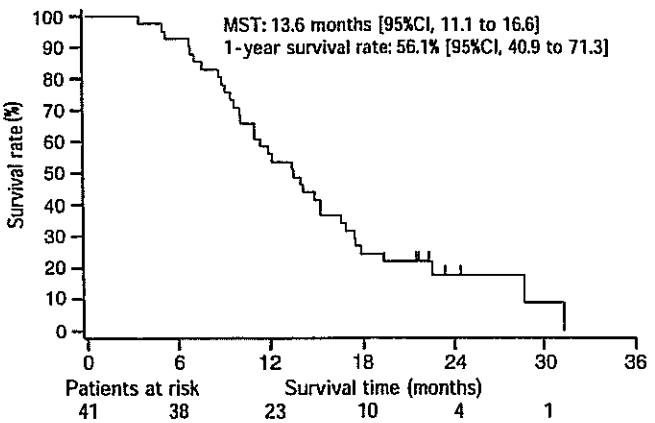

Figure 1. Overall survival of patients with extensive-stage small-cell lung cancer who were treated with amrubicin and cisplatin at the recommended dose. MST, median survival time; 95% CI, 95% confidence interval.

Toxicity in patients treated at the RD

The worst grades of hematological and non-hematological toxicities experienced by each patient are listed in Table 7. Hematological toxicity, especially leukopenia and neutropenia, was common and relatively severe. Grade 3 or worse leukopenia and neutropenia occurred in 65.9% and 95.1% of patients, respectively. Febrile neutropenia was observed in two patients at level 2. Grade 3 or worse anemia and thrombocytopenia occurred in 53.7% and 24.4% of patients, respectively. Four patients received platelet transfusions. Common non-hematological toxicities were gastrointestinal toxicity, such as anorexia, nausea, vomiting, constipation, diarrhea and stomatitis. Gastric ulcers developed in three patients. Hepatic and renal toxicity were not common in this study. Grade 3 or worse hyponatremia and hypokalemia occurred in 22% and 9.8% of patients, respectively. One patient developed myocardial infarction; however, cardiac toxicity was not common. No treatment-related deaths were observed.

Discussion

Doxorubicin and epirubicin are classified as active agents for SCLC, for which single-agent activity is a >20% response rate [19]. Doxorubicin has been used as a constituent of combination therapy for SCLC in the CAV (cyclophospamide, doxorubicin and vincristine) and CAP (cyclophosphamide, doxorubicin and cisplatin) regimens. Epirubicin has shown

Table 7. Toxicity in patients treated at the recommended dose ($n=41$)

	Grade (NCI CTC)					Grade 3/4 (%)
	0	1	2	3	4	
Leukopenia	1	0	13	20	7	65.9
Neutropenia	0	1	1	7	32	95.1
Febrile neutropenia	41	–	–	0	0	0.0
Hemoglobin	1	8	10	17	5	53.7
Thrombocytopenia	9	14	8	10	0	24.4
Stomatitis	22	13	5	1	0	2.4
Anorexia	1	14	13	13	0	31.7
Nausea	3	15	14	9	0	22.0
Vomiting	20	8	11	2	0	4.9
Constipation	24	1	13	3	0	7.3
Diarrhea	26	12	1	2	0	4.9
Gastric ulcer	38	0	1	2	0	4.9
Bilirubin	24	12	4	1	0	2.4
Hyponatremia	18	14	–	7	2	22.0
Hypokalemia	31	6	–	4	0	9.8
Hyperkalemia	33	3	4	1	0	2.4
Hypocalcemia	31	5	4	0	1	2.4

NCI CTC, National Cancer Institute Common Toxicity Criteria.

50% and 48% response rates in two clinical studies in 41 and 80 previously untreated patients, respectively, with ED-SCLC [20, 21]. However, currently, combination modalities containing doxorubicin or epirubicin are not being used in the therapy of SCLC, in preference to combination therapy with cisplatin and etoposide. Since amrubicin has shown excellent single-agent activity [15], it can be expected to be superior to other anthracyclines in the treatment of SCLC. Additionally, the present results of combination therapy with cisplatin support the view that amrubicin may be a promising agent that overcomes the therapeutic plateau of SCLC.

Amrubicin is one of the most promising new agents for the treatment of SCLC. In a previous phase II study of amrubicin 45 mg/m^2 on days 1–3 every 3 weeks as a monotherapy for chemonaive ED-SCLC, a 76% overall response rate and 11.7 month MST were observed [15]. The overall response rate and MST were comparable to those achieved with standard combination chemotherapy, such as etoposide plus cisplatin [5, 6]. Moreover, only a few patients treated in the phase II study received salvage chemotherapy consisting of cisplatin and etoposide [15]. The major toxicity of amrubicin as a monotherapy was hematological toxicity: grade 4 leukopenia and neutropenia were seen in 12.1% and 39.4% of patients, respectively, and thrombocytopenia and anemia of grade 3 or worse in 21.2%. Hepatic, renal and cardiac toxicities with amrubicin were not common. Cisplatin is a key drug for the treatment of SCLC and its hematological toxicity, such as leukopenia and neutropenia, is not severe. Thus, we conducted a phase I–II study of amrubicin and cisplatin treatment for chemonaive ED-SCLC to determine the MTD of this combination therapy, to

assess the efficacy and safety of the drugs delivered at their RD in chemonaive ED-SCLC, and to examine pharmacokinetics.

The topoisomerase I inhibitor, irinotecan, is also very effective for SCLC [6]. Combinations of topoisomerase I and topoisomerase II inhibitors, such as irinotecan plus etoposide, have been reported as active combination chemotherapy for SCLC [22]. Thus, combination of irinotecan and amrubicin is another candidate for new combination chemotherapy for SCLC. A phase I study of irinotecan and amrubicin for chemonaive non-SCLC was performed in National Cancer Center Hospital (unpublished data). However, the MTD was less than irinotecan 60 mg/m^2 on days 1 and 8 and amrubicin 35 mg/m^2 on days 2–4, due to relatively severe myelotoxicity. We considered that amrubicin <35 mg/m^2 on days 2–4 with irinotecan 60 mg/m^2 on days 1 and 8 was insufficient to treat SCLC.

In this study, we determined the RD to be amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1 every 3 weeks, and 41 patients were treated at the RD. Main toxicities of this combination chemotherapy were myelosuppression, especially leukopenia and neutropenia, and gastrointestinal toxicities including anorexia, nausea, vomiting, constipation, diarrhea, stomatitis and gastric ulcer. Of 41 patients, 32 (78%) patients received four or more courses of chemotherapy, and 22 (54%) patients completed four courses of chemotherapy without dose modification. One patient developed myocardial infarction; however, other cardiac toxicity, including decrease in left ventricle ejection fraction, was not observed in up to six courses of chemotherapy. The total dose of amrubicin was 720 mg/m^2. Grade 3 or 4 hyponatremia occurred in nine (22%) patients; however, most of the patients were asymptomatic. No unexpected toxicities and no treatment-related deaths were observed in this study. Toxicities observed in this study were manageable.

Four CRs and 32 PRs occurred, for an objective response rate of 87.8% (95% CI 73.8% to 95.9%) in 41 patients treated at the RD. In most patients, ProGRP levels changed in parallel with tumor responses. The MST of the 41 patients was 13.6 months, and the 1-year survival rate was 56.1%. These results were better than recently reported results for irinotecan and cisplatin in chemonaive ED-SCLC: an objective response rate of 84% and MST of 12.8 months [6]. The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC. A possible reason for the better results is overselection of patients, because we used unusual exclusion criteria such as non-steroidal anti-inflammatory drug or adrenal cortical steroid use for >50 days, and gastric and/or duodenal ulcer. However, in a phase II study, this kind of bias is not uncommon.

Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin, alternating with cyclophosphamide, doxorubicin and vincristine, had been considered as standard chemotherapy for SCLC in North America and Japan. A Japanese phase III trial (JCOG 9511) demonstrated that treatment with four cycles of irinotecan plus cisplatin every 4 weeks yielded a highly significant improvement in survival in ED-SCLC patients over standard etoposide plus cisplatin, with less myelosuppression [6]. Based on the results of the JCOG 9511 trial, irinotecan plus cisplatin is considered to be the reference chemotherapy arm for ED-SCLC in future trials in Japan [23]. The JCOG are preparing a phase III clinical trial of amrubicin and cisplatin for previously untreated ED-SCLC to compare combination therapy of irinotecan with cisplatin.

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Prediction of sensitivity of advanced non-small cell lung cancers to gefitinib (Iressa, ZD1839)

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Gefitinib (Iressa, ZD1839), an inhibitor of epidermal growth factor receptor-tyrosine kinase, has shown potent anti-tumor effects and improved symptoms and quality-of-life of a subset of patients with advanced non-small cell lung cancer (NSCLC). However, a large portion of the patients showed no effect to this agent. To establish a method to predict the response of NSCLC patients to gefitinib, we used a genome-wide cDNA microarray to analyze 33 biopsy samples of advanced NSCLC from patients who had been treated with an identical protocol of second to seventh line gefitinib monotherapy. We identified 51 genes whose expression differed significantly between seven responders and 10 non-responders to the drug. We selected the 12 genes that showed the most significant differences to establish a numerical scoring system (GRS, gefitinib response score), for predicting response to gefitinib treatment. The GRS system clearly separated the two groups without any overlap, and accurately predicted responses to the drug in 16 additional NSCLC cases. The system was further validated by the semi-quantitative RT–PCR, immunohistochemistry and ELISA for serological test. Moreover, we proved that the anti-apoptotic activity of amphiregulin, a protein that was significantly over-expressed in non-responders but undetectable in responders, leads to resistance of NSCLC cells to gefitinib *in vitro*. Our results suggested that sensitivity of a given NSCLC to gefitinib can be predicted according to expression levels of a defined set of genes that may biologically affect drug sensitivity and survival of lung cancer cells. Our scoring system might eventually lead to achievement of personalized therapy for NSCLC patients.

INTRODUCTION

Lung cancer, the leading cause of cancer death worldwide, is a major health problem in many countries. Chemotherapy is the mainstay for treatment of this disease; surgery is rarely indicated because by the time of diagnosis the majority of lung tumors have reached locally advanced stage III (44%) or metastatic stage IV (32%) (1). Nevertheless, a large meta-analysis revealed that platinum-based chemotherapy prolongs for only about 6 weeks the median survival time of patients with advanced non-small cell lung cancer (NSCLC), compared with the best supportive care (2). Within the last decade, a number of new cytotoxic agents such as paclitaxel, docetaxel, gemcitabine and vinorelbine have emerged to offer

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multiple choices for patients with advanced lung cancer. However, each of those regimens confers only a modest survival benefit compared with cisplatin-based therapies (3,4). To overcome these limitations, new therapeutic strategies that rely on agents designed to target specific tumor-associated molecules are under development (5,6).

Gefitinib (Iressa, ZD1839) is an orally administered inhibitor of epidermal growth factor receptor-tyrosine kinase (EGFR-TK), an enzyme involved in certain signaling pathways that drive proliferation, invasion and survival of cancer cells (7). Gefitinib has shown potent anti-tumor effects and brought about rapid improvements in NSCLC-related symptoms and quality of life among some patients with advanced NSCLC, who had not responded to platinum-based chemotherapy. In a randomized, double-blind phase II monotherapy trial (the IDEAL 1 trial), the use of gefitinib as a second or third line of chemotherapy achieved tumor-response rates of 18.4% (95% CI: 11.0–25.9%) for advanced NSCLCs; in the IDEAL 2 trial, this drug as the third or fourth line of chemotherapy achieved 11.8% (95% CI: 6.2–19.7%) tumor response (8–10). Moreover, in these trials the drug achieved high rates of disease control (54.4% in IDEAL 1, 42.2% in IDEAL 2) and overall improvement in symptoms (40.3% in IDEAL 1, 43.1% in IDEAL 2). The results were promising when compared with responses to conventional cytotoxic agents, but about half of the patients enrolled in these studies showed no improvement in symptoms and in some cases the medication caused serious adverse effects, including life threatening ones such as interstitial pneumonia (11). The figures do indicate considerable potential for improving prognosis and quality of life for many patients with advanced NSCLC, if we could match treatments to individual cases by using this type of drug more effectively. One approach to that goal is to identify 'cancer profiles' of individual NSCLCs and determine in advance which tumors are likely to respond to gefitinib.

In the study reported here, we applied a cDNA microarray system representing 27 648 genes to select a defined set of genes that could predict responsiveness of advanced NSCLCs to gefitinib. Statistical analysis of expression profiles in 17 clinical samples identified dozens of genes that were differentially expressed between gefitinib-responders and non-responders. A gefitinib-response scoring (GRS) system based on expression patterns of a selected set of those genes successfully predicted the response to gefitinib therapy among additional 16 NSCLC samples. The data was further validated with semi-quantitative RT–PCR, immunohistochemistry and ELISA, implying possible application of our system to practical clinical tests. A gefitinib-sensitivity assay *in vitro* brought to light at least one biological mechanism of gefinitib-resistance of NSCLC cells, i.e. induction of resistance by amphiregulin (AREG). This protein was significantly up-regulated in non-responders, but was not expressed in responders.

RESULTS

Response to gefitinib treatment

Of the 53 patients enrolled in this trial, 46 had tumors diagnosed as adenocarcinomas (86.8%), five were squamous-cell carcinomas (9.4%), two were large-cell carcinomas (3.8%). Fifteen patients achieved a partial response (PR) and nobody revealed a complete response (CR); 17 patients were classified as stable disease (SD) and 19 as progressive disease (PD). No clinical-response data were available for two of the patients. The tumor-response rate (CR + PR/CR + PR + SD + PD) for this treatment was 29.4%, and the disease control rate (CR + PR + SD/CR + PR + SD + PD) was 62.8% (Table 1).

Table 1. Summary of baseline patient characteristics

Characteristics	Percentage (%)	Number of patients
Sex		
Male	58.5	31
Female	41.5	22
Age		
Median	59	
Range	35–80	
Histology		
Adenocarcinoma	86.8	46
Squamous-cell carcinoma	9.4	5
Large-cell carcinoma	3.8	2
Stage		
IIIA	1.9	1
IIIB	7.5	4
IV	90.6	48
Performance status		
0	26.4	14
1	60.4	32
2	13.2	7
Number of prior regimen		
1	24.5	13
2	35.9	19
3	28.3	15
4	0	0
5	7.5	4
6	3.8	2
Response to gefitinib therapy		
CR	0	0
PR	28.3	15
SD	32.1	17
PD	35.8	19
Unknown	3.8	2
Tumor response rate (CR + PR/CR + PR + SD + PD)	29.4	15
Disease control rate (CR + PR + SD/CR + PR + SD + PD)	62.8	32

Tumor samples were collected from 43 patients. Samples from 32 of those 43 contained sufficient numbers of cancer cells for analysis of expression profiles on our cDNA microarray. The numbers of samples that were judged to be suitable for further microarray analysis were 8 for PR, 7 for SD and 13 for PD (Table 2 and Fig. 1). Of the 28 samples, 17 were analyzed as learning cases (seven for PR and 10 for PD) and 11 were test cases (one for PR, three for PD and seven for SD) for establishing a predictive scoring system for the efficacy of gefitinib treatment. For further validation of the prediction system, another blinded set of samples from five newly

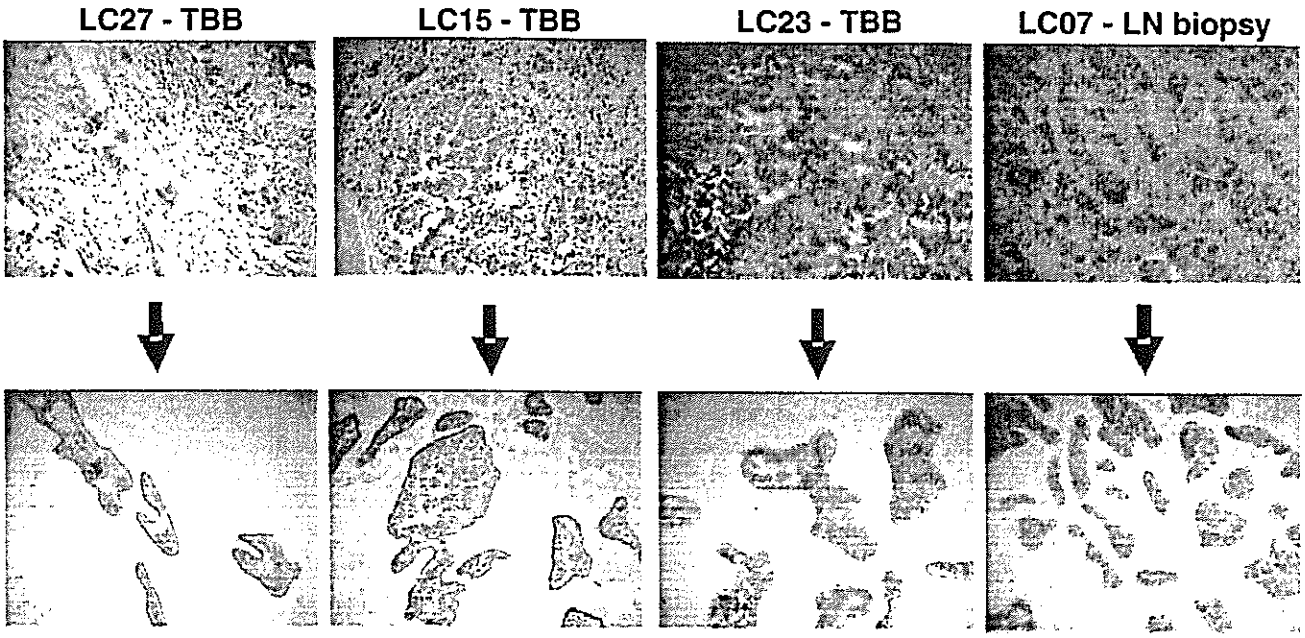

Figure 1. Images illustrating laser-microbeam microdissection of four representative lung adenocarcinomas. The upper row shows the samples before dissection; the lower row, dissected cancer cells (hematoxylin and eosin stain 100×). TBB indicates transbronchial biopsy; LN, lymph-node.

enrolled test-cases (four for PD and one for SD) were obtained and added finally to the initial 11 test cases.

Expression of EGFR and AKT

To determine the status of EGFR and AKT, a downstream effector molecule of EGFR in tumor tissue samples for microarray analysis, we carried out immunohistochemical staining with anti-EGFR, anti-phospho EGFR (p-EGFR), anti-AKT and anti-phospho AKT (p-AKT) antibodies. As shown in Table 7, high levels of EGFR, p-EGFR, AKT and p-AKT protein expression was detected in most NSCLC samples examined, but no correlation between any of these protein expression and sensitivity to gefitinib was observed ($P = 0.999$, 0.622, 0.999 and 0.546, respectively, Fisher's exact test).

Identification of genes associated with sensitivity to gefitinib

We attempted to extract genes that were differentially expressed between tumors from seven patients in the PR group (defined as responders) and those from 10 patients in the PD group (defined as non-responders) by comparing expression levels of 27 648 genes (Tables 2 and 3).

We carried out a random permutation test to distinguish between the two subclasses defined by tumor response, and identified 51 genes whose permutational P-values were less than 0.001 (Table 4). Expression levels of 40 genes were higher, and those of the other 11 were lower, in the non-responders.

Table 2. Number of cases suitable for analysis and their best overall responses

Number of cases	Best overall response				
	PR	SD	PD	Unknown	Total
All cases enrolled	15	17	19	2	53
Cases that consented to the study	15	14	13	1	43
Cases suitable for analysis	8	10	13	1	32
Learning cases[a]	7	0	10	0	17
Test cases[a,b]	1	7	3	0	11

[a]Learning cases were used for developing the GRS, whereas test cases were used for validation.
[b]Another blinded set of samples from five newly enrolled cases were also added to the tests later.

Establishment of a predictive scoring system for the efficacy of gefitinib treatment

On the basis of the expression profiles of the 51 genes selected, we tried to establish a predictive scoring system for the efficacy of gefitinib treatment. Prediction scores, termed GRS, were calculated according to procedures described previously (see Materials and Methods). To determine the number of candidates that provided the best separation of the two groups, we ranked the 51 genes on the basis of the significance of their permutational P-values and calculated prediction scores by the leave-one-out test, in decrements of one starting from the bottom of the rank-ordered list (51, 50, 49, 48, etc.). We calculated a classification score (CS), a standard we had previously defined for evaluation of the ability to discriminate two classes, for each set of genes.

Table 3. Clinicopathological features of patients

Case no.[a]	Sex	Age	Histology type[b]	T	N	M	Stage classification[c]	Number of previous chemotherapy	EGFR stained tumour cell (%)	Plasma gefitinib concentration (ng/ml)	Response to gefitinib[d] 1st month	2nd month	3rd month	4th month	Best overall response[e]	Use for prediction[f]	GRS[g]
LC01	Female	36	ADC	1	0	1	IV	1		258.9	PR	PR	PR	PR	PR	Learning	100
LC02	Male	64	ADC	2	3	1	IV	3	80	140.3	PR	PR	PR	PR	PR	Learning	100
LC03	Female	54	ADC	2	0	1	IV	3	80	167.0	PR	PR	PR	PR	PR	Learning	100
LC04	Female	75	ADC	2	1	1	IV	1	20	169.7	PR	PR	PR	PR	PR	Learning	100
LC05	Female	73	ADC	0	2	1	IV	5	30	300.6	PR	PR	PR	PR	PR	Learning	100
LC06	Female	75	ADC	4	1	1	IV	3		874.0	SD	PR	PR	PR	PR	Learning	100
LC07	Female	70	ADC	2	1	1	IV	3	80	460.8	SD	PR	PR	PR	PR	Learning	100
LC08	Female	47	ADC	4	3	1	IV	2	95	306.5	PR	PR	PR	PR	PR	Test	54.8
Mean (range)		62 (36–75)						2.6 (1–5)	64 (20–95)	334.7 (140.3–874.0)							
LC09	Female	63	ADC	4	0	1	IV	3	90	743.4	SD	SD	SD	SD	SD	Test	61.6
LC10	Male	56	ADC	2	0	1	IV	6	70	511.8	SD	SD	SD	SD	SD	Test	−9.8
LC11	Male	67	ADC	4	0	1	IV	2	0	631.3	SD	SD	SD	SD	SD	Test	−5.3
LC12	Male	53	ADC	4	3	1	IV	2		306.1	SD	SD	SD	PD	SD	Test	−23.8
LC13	Female	56	ADC	4	2	0	IIIB	2	40	364.8	SD	SD	PD		SD	Test	−58.5
LC14	Female	62	ADC	4	2	1	IV	3	60	322.4	SD	SD	PD		SD	Test	−83
LC15	Male	61	ADC	0	0	1	IV	5	60	278.9	SD	SD	PD		SD	Test	−40.5
Mean (range)		60 (53–67)						3.3 (2–6)	53 (0–90)	451.2 (278.9–631.3)							
LC16	Male	42	ADC	4	3	1	IV	5	90	212.6	SD	PD			PD	Learning	−63.9
LC17	Female	54	ADC	2	3	1	IV	2	50	320.6	SD	PD			PD	Learning	−86
LC18	Female	61	ADC	1	3	0	IIIB	2		229.3	SD	PD			PD	Learning	−67.8
LC19	Male	59	ADC	0	2	1	IV	2	30	150.7	SD	PD			PD	Learning	−57.1
LC20	Male	65	ADC	0	3	1	IV	3		167.8	SD	PD			PD	Learning	−59.1
LC21	Male	55	ADC	4	3	1	IV	3	80		PD				PD	Learning	−73.1
LC22	Male	80	ADC	4	3	1	IV	2	80		PD				PD	Learning	−55.5
LC23	Male	35	ADC	4	0	1	IV	5			PD				PD	Learning	−100
LC24	Male	57	ADC	4	3	1	IV	1	0		PD				PD	Learning	−46.7
LC25	Female	65	ADC	2	0	1	IV	1		356.3	PD				PD	Learning	−86.1
LC26	Male	64	SCC	3	3	1	IV	2		405.6	SD	PD			PD	Test	-67.7
LC27	Female	65	ADC	4	2	1	IV	1			PD				PD	Test	−69.4
LC28	Male	74	ADC	2	1	1	IV	1	10		PD				PD	Test	−64.8
Mean (range)		60 (35–80)						2.3 (1–5)	49 (0–90)	263.2 (150.7–405.6)							

[a]For further validation of the GRS, another blinded set of samples from five newly enrolled cases (four PD and one SD) were also added to these 28 cases later.
[b]ADC, adenocarcinoma; SCC, squamous-cell carcinoma.
[c]TNM clinical classification and stage grouping were assessed based on the UICC/WHO classification.
[d]Objective tumor response to gefitinib was assessed every 4 weeks after the start of treatment using UICC/WHO Criteria. PR, partial response; SD, stable disease; PD, progressive disease.
[e]Overall best response was evaluated based on the definitions as mentioned in Materials and Methods.
[f]Learning, samples used for developing the GRS; Test, samples used for validation of the GRS.
[g]GRS, gefitinib response score determined by prediction system.

Table 4. List of 51 candidate genes for discriminating responder (PR) from non-responder (PD) to gefitinib[a]

Rank order	GenBank accession no.	Symbol	Gene name	Predominantly expressed class	Permutational *P*-value	Median-fold difference (log 2)
1	NM_024829	*FLJ22662*	Hypothetical protein FLJ22662	PD	8.1×10^{-12}	2.0
2	BC009799	*AREG*	Amphiregulin (schwannoma-derived growth factor)	PD	9.3×10^{-12}	8.0
3	NM_014325	*CORO1C*	Coronin, actin binding protein, 1C	PD	2.3×10^{-10}	4.6
4	BC010488	*AVEN*	Apoptosis, caspase activation inhibitor	PD	4.2×10^{-10}	4.3
5	NM_004090	*DUSP3*	Dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	PD	9.4×10^{-10}	4.4
6	AI026836	*DJ473B4*	Hypothetical protein dJ473B4	PD	1.7×10^{-9}	8.0
7	BU500509	*PHLDA2*	Pleckstrin homology-like domain, family A, member 2	PD	1.8×10^{-9}	8.0
8	NM_016090	*RBM7*	RNA binding motif protein 7	PD	1.8×10^{-8}	2.9
9	BX092512		EST	PD	7.7×10^{-8}	3.0
10	AI436027	*OSMR*	Oncostatin M receptor	PD	1.1×10^{-7}	3.7
11	AI971137	*GCLC*	Glutamate–cysteine ligase, catalytic subunit	PD	1.2×10^{-7}	3.9
12	BQ024877	*COL4A3BP*	Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	PD	2.0×10^{-7}	3.6
13	U52522	*ARFIP2*	ADP-ribosylation factor interacting protein 2 (arfaptin 2)	PD	2.6×10^{-7}	2.8
14	BM996053	*C10orf9*	Chromosome 10 open reading frame 9	PD	4.2×10^{-7}	2.5
15	AK025452	*NIP30*	NEFA-interacting nuclear protein NIP30	PD	5.1×10^{-7}	3.7
16	N52048	*KIAA0776*	KIAA0776 protein	PD	5.4×10^{-7}	7.2
17	AA507009	*SLC35F2*	Solute carrier family 35, member F2	PD	6.0×10^{-7}	5.8
18	AA226243	*GAMLG*	Calcium modulating ligand	PD	6.8×10^{-7}	5.0
19	AF005888	*NOC4*	Neighbor of COX4	PD	1.1×10^{-6}	4.0
20	AF012281	*PDZK1*	PDZ domain containing 1	PD	1.3×10^{-6}	4.5
21	AI188190	*DIS3*	Mitotic control protein dis3 homolog	PD	1.7×10^{-6}	3.8
22	BC001535	*CGI-48*	CGI-48 protein	PD	2.0×10^{-6}	3.5
23	NM_007007	*CPSF6*	Cleavage and polyadenylation specific factor 6, 68 kDa	PD	2.2×10^{-6}	3.4
24	NM_002254	*KIF3C*	Kinesin family member 3C	PD	2.2×10^{-6}	3.5
25	BQ135232	*CD9*	CD9 antigen (p24)	PD	2.2×10^{-6}	1.7
26	BC051322	*LRRC8*	Leucine rich repeat containing 8	PD	2.5×10^{-6}	3.4
27	BC038504	*SNF1LK*	SNF1-like kinase	PD	2.6×10^{-6}	2.8
28	U78556	*CRA*	Cisplatin resistance associated	PD	2.7×10^{-6}	3.7
29	BC035625	*EGR2*	Early growth response 2 (Krox-20 homolog, *Drosophila*)	PD	3.4×10^{-6}	3.0
30	X52426	*KRT13*	Keratin 13	PD	1.9×10^{-5}	3.4
31	NM_005504	*BCAT1*	Branched chain aminotransferase 1, cytosolic	PD	2.3×10^{-5}	1.7
32	NM_006643	*SDCCAG3*	Serologically defined colon cancer antigen 3	PR	2.6×10^{-5}	3.7
33	AA464095	*PIGK*	Phosphatidylinositol glycan, class K	PD	3.2×10^{-5}	1.1
34	AA961188	*MRPS9*	Mitochondrial ribosomal protein S9	PD	9.8×10^{-5}	2.3
35	NM_018123	*ASPM*	asp (abnormal spindle)-like, microcephaly associated (*Drosophila*)	PR	2.3×10^{-4}	2.8
36	NM_022735	*ACBD3*	acyl-Coenzyme A binding domain containing 3	PD	2.4×10^{-4}	3.8
37	AA160544	*ZNF325*	Zinc finger protein 325	PR	2.7×10^{-4}	4.5
38	AK057653	*LOC285513*	Hypothetical protein LOC285513	PD	2.7×10^{-4}	3.8
39	NM_003310	*TSSC1*	Tumor suppressing subtransferable candidate 1	PD	2.9×10^{-4}	4.7
40	BC007451	*XAB1*	XPA binding protein 1	PD	3.0×10^{-4}	1.3
41	BC035467	*HNLF*	Putative NFkB activating protein HNLF	PR	3.5×10^{-4}	1.1
42	CK004097	*EIF4EBP2*	Eukaryotic translation initiation factor 4E binding protein 2	PR	3.6×10^{-4}	1.4
43	NM_144683	*MGC23280*	Hypothetical protein MGC23280	PR	4.2×10^{-4}	2.3
44	NM_004600	*SSA2*	Sjogren syndrome antigen A2 (60 kDa, ribonucleoprotein autoantigen SS-A/Ro)	PR	4.2×10^{-4}	1.2
45	NM_002730	*PRKACA*	Protein kinase, cAMP-dependent, catalytic, alpha	PR	5.0×10^{-4}	1.2
46	NM_005102	*FEZ2*	Fasciculation and elongation protein zeta 2 (zygin II)	PD	6.1×10^{-4}	3.3
47	NM_005839	*SRRM1*	Serine/arginine repetitive matrix 1	PR	7.0×10^{-4}	1.4
48	NM_006207	*PDGFRL*	Platelet-derived growth factor receptor-like	PD	7.0×10^{-4}	2.4
49	AI096936	*SNX13*	Sorting nexin 13	PR	8.4×10^{-4}	1.6
50	NM_014785	*KIAA0258*	KIAA0258 gene product	PD	8.9×10^{-4}	2.5
51	BF973104	*TOM7*	Homolog of Tom7 (*S. cerevisiae*)	PR	1.0×10^{-3}	1.5

[a]The top 12 and 51 gene sets were listed as the rank-order of permutational *P*-values that were < 0.001.

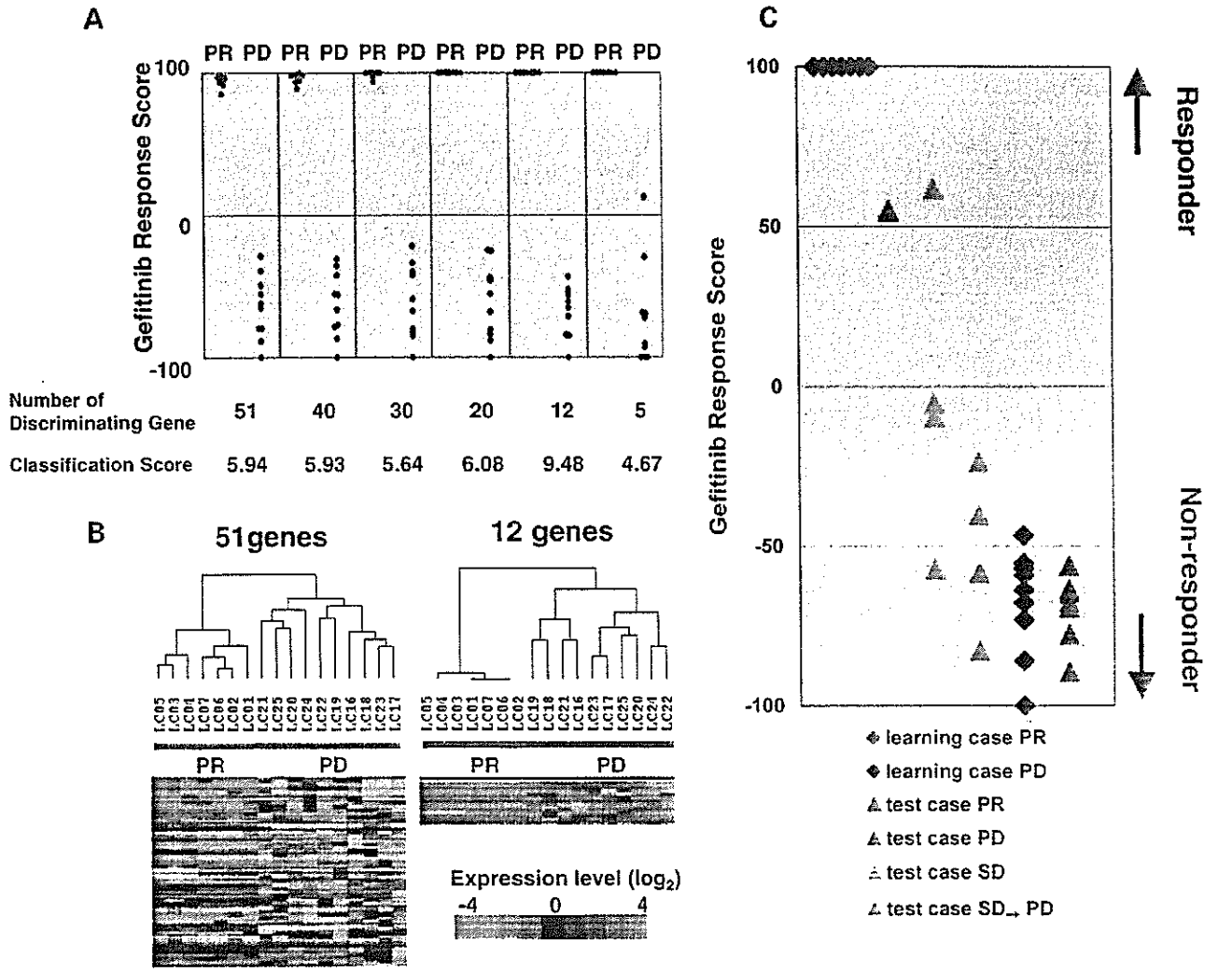

Figure 2. Establishing a scoring system to predict the efficacy of gefitinib treatment. (A) Different prediction scores appear when the number of discriminating genes is changed. The number of the discriminating gene sets (5–51) corresponds to the number of selected genes from the top of the rank-ordered list in Table 4. A larger value of classification score (CS) indicates better separation of the two groups. (B) Hierarchical clustering of 17 'learning' cases using 51 candidate genes for gefitinib sensitivity (left), and 12 prediction genes that were finally selected for the GRS (right). The dendrograms represent similarities in expression patterns among individual cases; longer branches indicate greater differences. The two groups were most clearly separated by the 12-gene set. (C) Schematic distinction of responder, non-responder and 'test cases' verified on the basis of the GRS. Red diamonds denote prediction scores for learning PR cases and blue diamonds represent learning PD cases. A pink triangle indicates a test PR case that had not been used for establishing GRS and blue triangles indicate test PD cases. Yellow triangles indicate test SD cases that kept the SD status throughout the 4 month observation period and green triangles indicate test cases once judged as SD at a certain time point of the study but showed progression of the disease within 3 or 4 months after the start of treatment.

As shown in Figure 2A, we obtained different prediction scores when the number of discriminating genes was changed. We obtained the best CS, meaning the best separation of responders from non-responders, when we calculated the scores using only the 12 top-ranked genes in our candidate list.

Hierarchical clustering analyses using all 51 genes, or only the top 12, classified all 17 cases into one of two groups according to the response to gefitinib (Fig. 2B). The two groups were most clearly separated when we used the top 12 genes for cluster analysis. Finally, we established a numerical drug-response-scoring algorithm that might be clinically applicable for predicting sensitivity of an individual NSCLC to gefitinib, on the basis of expression levels of the 12 selected genes.

To validate this prediction system, we investigated eight additional ('test') NSCLC cases (one for PR and seven for PD) that were completely independent of the 17 'learning' cases used for establishing the system. We examined gene-expression profiles in each of those samples and then calculated GRS on the basis of the expression levels of the 12 discriminating genes. As shown in Figure 2C, scores obtained by the GRS system were concordant with the clinical responses to gefitinib in all eight 'test' cases.

Table 5. Correlation of cDNA microarray data with RT–PCR

Rank order	Gene symbol	Spearman rank correlation ρ	P-value
1	*FLJ22662*	0.69	0.02
2	*AREG*	0.53	0.08
3	*CORO1C*	0.35	0.24
4	*AVEN*	0.63	0.04
5	*DUSP3*	0.63	0.04
6	*DJ473B4*	0.45	0.14
7	*PHLDA2*	0.84	0.01
8	*RBM7*	0.83	0.01
9	*EST(BX092512)*	0.63	0.04
10	*OSMR*	0.67	0.03
11	*GCLC*	0.46	0.13
12	*COL4A3BP*	0.27	0.24

Correlations positive for all 12 genes and significantly positive for seven of 12.

Table 6. Result of immunohistochemical staining for prediction markers

	PR	PD
AREG	1/5	5/6
TGFA	2/5	6/6
ADAM9	1/5	4/6
CD9	2/5	5/6
OSMR	2/5	6/6

Table 7. Result of immunohistochemical staining for EGFK and AKT

	PR	PD
EGFR	6/6	6/7
p-EGFR	5/7	5/9
p-AKT	4/5	4/6
AKT	4/6	4/6

GRS values for patients with SD in tumor response

GRS values for the eight test-SD patients were calculated according to the predictive scoring system established earlier. Although the values were widely distributed from −83.0 (predicted as non-responder) to 61.6 (responder), the scores of patients who retained SD status throughout the observation period were likely to be higher than those of patients who had been judged as SD at a certain time-point of the study but showed progression of the disease within 3 or 4 months after the start of treatment (Fig. 2C). Although the GRS system was established on the basis of gene-expression profiles that distinguished between patients with PR and patients with PD (without SD) in tumor response, these results suggested the possibility that the GRS may serve in classifying SD patients into groups according to their response to gefitinib.

Validation of GRS with semi-quantitative RT–PCR analysis

To confirm differential expression of the top 12 predictive genes between PR and PD cases, expression values derived from microarray data were correlated with values from semi-quantitative RT–PCR of RNAs from the same patients (five PR and seven PD) (Table 5 and Fig. 3A). Spearman rank correlations were positive for all of the 12 genes and significantly positive for seven of 12 genes.

Immunohistochemical validation of GRS

To validate differential expression of the predictive protein markers between PR and PD cases, we carried out immunohistochemical staining with five different antibodies for AREG, TGFA, ADAM9, CD9 and OSMR, all of which were known to be involved in the ligand-EGFR signaling and whose permutational P-values were < 0.01 (Supplementary Material, Table S1). We first stained paired tumor tissue sections obtained by TBB and lymph-node biopsy from the same patients using these five antibodies. No intra-patient differences on protein expression of these five markers were observed in three different patients (Fig. 3B). We also validated the microarray data with the five markers in 11 NSCLC samples (five for PR and six for PD). The results were consistent with the microarray data (Table 6 and Fig. 3C).

Serum levels of TGFA

To further evaluate the availability of the prediction system in routine clinical situations, we detected TGFA protein which was known to be the ligand for EGFR and whose permutational P-values were < 0.01, using ELISA in serum samples from five PR, 10 SD and 20 PD patients that were independently collected for serological test and were not enrolled in microarray analysis. The serum levels of TGFA were 19.0 ± 2.8 pg/ml (mean $\pm$ SE) in PD patients, 13.9 ± 1.9 pg/ml in SD patients and 12.8 ± 1.4 pg/ml in PR patients (Fig. 4). Twelve of 20 serum samples from PD patients were positive for TGFA and all samples from PR patients were negative, when 16.0 pg/ml was used as a cutoff.

In vitro gefitinib treatment and AREG-autocrine assay

AREG, a ligand for EGFR and other ERBB members, was significantly over-expressed in non-responders but not (or hardly) detectable in responders. To investigate whether AREG protein leads to resistance of NSCLCs to gefitinib therapy when it is secreted in an autocrine manner, we performed the following biological analyses. We initially identified expression of AREG mRNA in lung-adenocarcinoma cell lines NCI-H358 and -H522, but not in PC-9, by means of RT–PCR experiments (Fig. 5A). Next, we performed flow-cytometric analysis 72 h after treatment of PC-9 cells with 1.0 μM of gefitinib, and found that gefitinib increased the percentages of nuclei in sub-G_1 (24%) compared with cells with no treatment (6%) (data not shown). This result suggested that gefitinib might induce apoptosis in PC-9 cells.

We then analyzed the viability of PC-9 cells, which are gefitinib-sensitive and do not express *AREG*, after culture in serum-free medium or in serum-free, conditioned medium

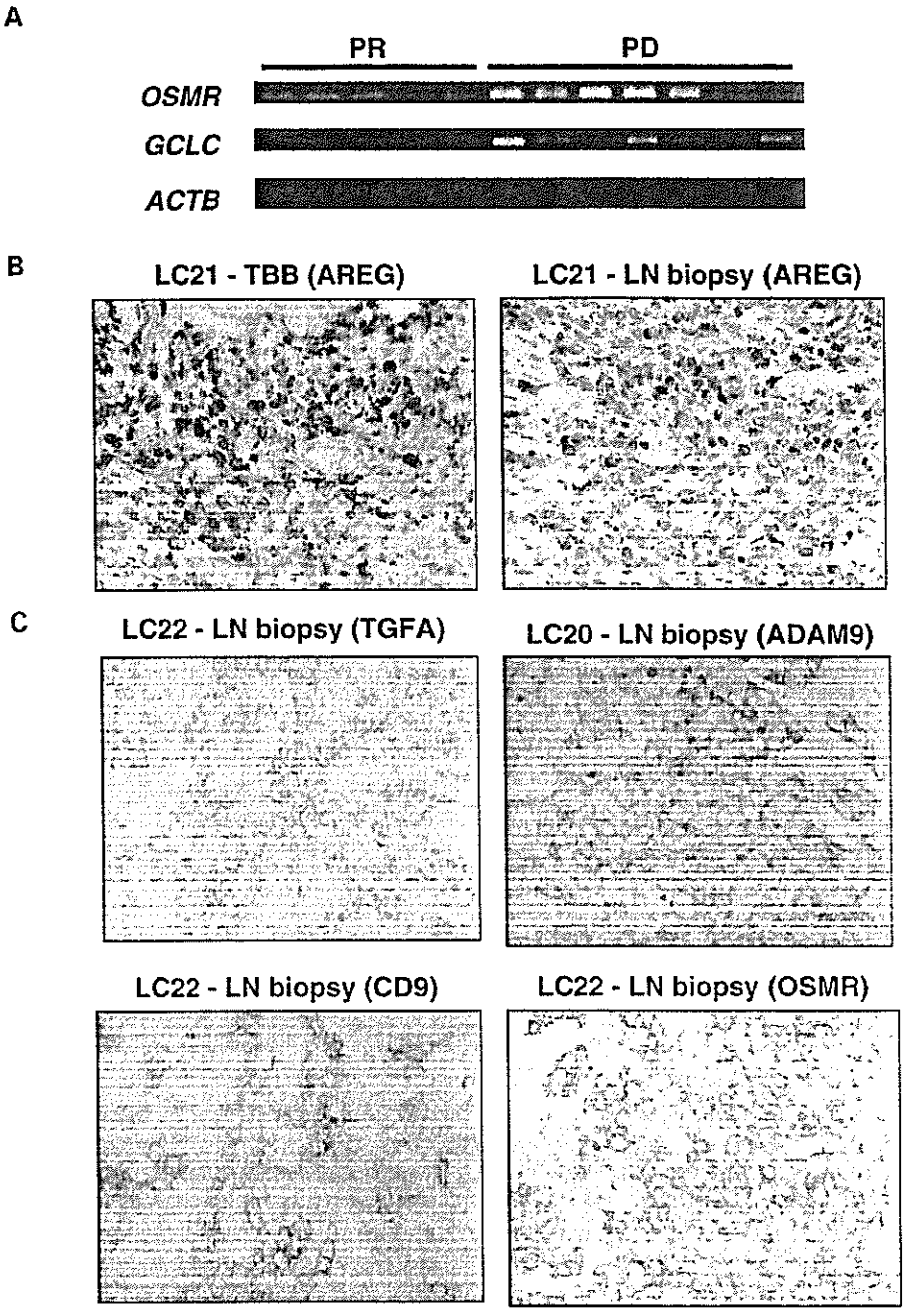

Figure 3. Validation of GRS with semi-quantitative RT–PCR and immunohistochemical analyses. (A) Representative image of semi-quantitative RT–PCR analysis of RNAs from the PR and PD groups. *OSMR* and *GCLC* genes were over-expressed in non-responders (PD). The integrity of each cDNA template was controlled through amplification of *ACTB*. (B) Immunohistochemical staining of representative samples from fiberscopic transbronchial biopsy (TBB) and lymph-node (LN) biopsy from the same PD-patient (no. LC21), using anti-AREG antibody (×200). (C) Immunohistochemical staining of representative samples from PD patients, using antibodies for other four prediction markers (TGFA, ADAM9, CD9 and OSMR) (×200).

obtained from NCI-H358 or -H522 cells grown in the presence or absence of 0.5 or 1.0 μM of gefitinib. As shown in Figure 5B, the viability of PC-9 cells incubated in the serum-free, conditioned medium containing gefitinib was greater than that of PC-9 cells grown in serum-free medium with the same concentrations of gefitinib.

To investigate whether AREG, secreted in an autocrine manner, inhibits apoptosis of NSCLC cells treated with gefitinib, we cultured PC-9 cells in serum-free medium containing recombinant AREG protein at final concentrations of 1–100 ng/ml, in the presence or absence of 1.0 μM gefitinib. The viability of PC-9 cells incubated with both AREG and

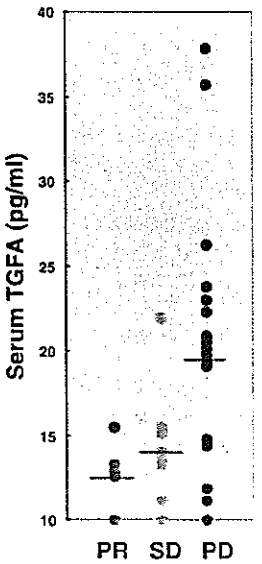

Figure 4. Serologic concentration of TGFA determined by ELISA in five PR, 10 SD and 20 PD adenocarcinoma cases. The averaged serum levels of TGFA were shown as black bars: 19.0 ± 2.8 pg/ml (mean ± SE) in PD patients, 13.9 ± 1.9 pg/ml in SD patients, and 12.8 ± 1.4 pg/ml in PR patients.

1.0 μM gefitinib was increased when compared with cells incubated with 1.0 μM gefitinib only, in an AREG dose dependent manner (Fig. 5C). On the other hand, recombinant AREG alone had no effect on the viability of PC-9 cells (Fig. 5C). This observation appeared to indicate that AREG inhibits the apoptosis induced by gefitinib, but does not in itself affect cell viability.

DISCUSSION

A large body of evidence supports the view that molecules in the EGFR autocrine pathway are involved in a number of processes important to cancer formation and progression, including cell proliferation, angiogenesis and metastatic spread (5). Therapeutic blockade of specific signaling, therefore, could be a promising strategy for cancer treatment. Gefitinib, a synthetic anilinoquinazoline, inhibits the tyrosine kinase activity of EGFR by competing with adenosine triphosphate for a binding site on the intracellular domain of the receptor (7). In phase II trials (IDEAL 1 and IDEAL 2), use of gefitinib as a second, third or fourth line monotherapy for advanced NSCLC achieved tumor-response rates of nearly 20% (8–10), which were superior to those achieved with conventional cytotoxic agents. Multivariate analysis of patients in the IDEAL 1 study suggested that the response rate in females might be higher than in males, and higher in patients with adenocarcinomas than in patients with squamous-cell carcinomas (odds ratios 2.7 and 3.5, respectively) (9). Recent study suggested that individuals in whom gefitinib is efficacious are more likely to have adenocarcinomas of the bronchioloalveolar subtype and to be never smokers (odds ratios 13.5 and 4.2, respectively) (12). The higher tumor-response rate (29.4%) documented in the clinical trial reported here might reflect a higher proportion of patients with adenocarcinoma (46 adenocarcinomas, five squamous-cell carcinomas and two large-cell carcinomas) than has been the case in other studies. The clinicopathological determinants of gefitinib sensitivity, including bronchioloalveolar carcinoma (BAC) features, are predictive to a certain extent (9,10,12,13); however, previous reports and our observations obviously suggest that no factors can perfectly predict the response of NSCLC to gefitinib treatment. It was also reported quite recently that somatic mutations of *EGFR* may predict sensitivity to gefitinib and mutant EGFRs selectively may activate AKT and STAT signaling *in vitro*, which transduce anti-apoptotic pathways (14–16); however, our mutational search proved that there is no significant correlation between the *EGFR* mutations and disease control effect of gefitinib therapy (PR + SD versus PD) (data not shown). Moreover, there is no evidence of correlation between response to gefitinib treatment and AKT/p-AKT protein level. We also did not identify transcriptional activation of the components of AKT/STAT signaling in the list of our prediction genes (top 132 genes; $P < 0.01$; Supplementary Material, Table S1). This result independently confirms no correlation between sensitivity to gefitinib and activation of AKT/STAT signaling. Therefore, novel methods to precisely discriminate responders from non-responders in advance could allow a more focused use of gefitinib in clinical settings.

By statistical analysis of gene-expression profiles of advanced NSCLCs obtained on cDNA microarrays, we identified dozens of genes associated with sensitivity to gefitinib. We introduced a prediction-scoring system based on expression of the 12 genes that had shown the most significant differences in expression levels between responder (PR) and non-responder (PD) groups. This set of genes was selected from expression profiles of lung adenocarcinomas; however, the GRS system successfully classified all eight of our 'test' PR and PD cases in accord with their clinical responses to gefitinib, and one of them was a squamous-cell carcinoma. Moreover, this system was likely to separate intermediate tumor responses (SD) into two groups, one representing patients who succeeded in maintaining the tumor-static effect for a long period and the other representing patients who failed to do so, although validation of the system in larger prospective trial is warranted.

In practical terms, we need to predict the chemosensitivity of individual tumors using the minimally invasive techniques available at every hospital, because patients with advanced NSCLCs are rarely candidates for surgical resection of their tumors. Therefore, we have tried to establish a prediction system that requires only the amount of cancerous tissue that can be obtained by, for example, flexible bronchofiberscopy. By verifying individual steps of the method, we were able to precisely profile gene expression in biopsy specimens as small as 1 mm. Relevant microarray results were confirmed by semi-quantitative RT–PCR for 12 genes that showed the most significant differences to establish a GRS system. Furthermore, we validated the effectiveness of antibodies for five different biomarkers (AREG, TGFA, ADAM9, CD9 and OSMR), all of which were reported to be involved in the ligand-EGFR signaling, for discriminating potential responders from non-responders, in both TBB and lymph-node

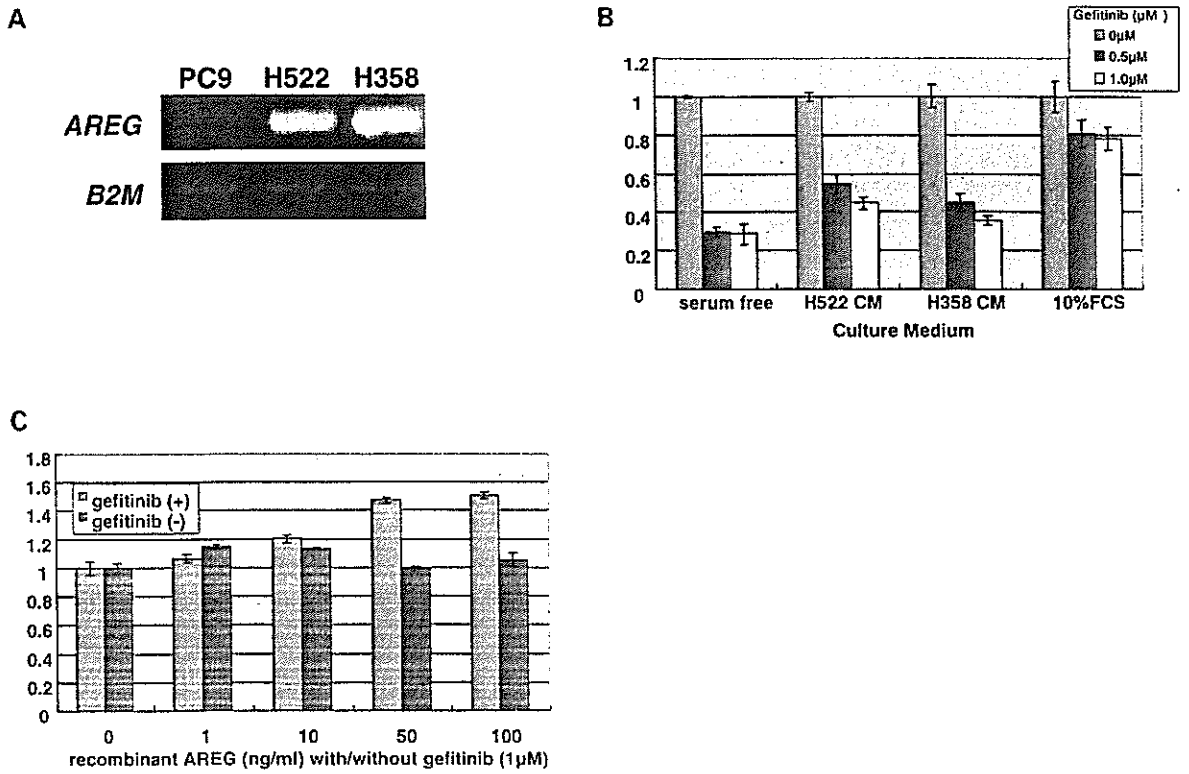

Figure 5. Anti-apoptotic effect of secreted AREG on gefitinib-sensitive PC-9 cells. (A) Expression of *AREG* transcript examined by semi-quantitative RT–PCR in lung adenocarcinoma cell lines PC-9, NCI-H358 and -H522. (B) PC-9 cells cultured in medium supplemented with 10% FCS, in serum-free medium or in serum-free conditioned medium (CM) obtained from cultures of NCI-H358 or -H522 cells. Each medium was replaced once with the same medium at the 48 h time point; 72 h after adding gefitinib at concentrations of 0.5 or 1.0 μM, cell viability was measured by MTT assays. The experiments were done in triplicate. The *y*-axis indicates the relative MTT value (MTT in the presence of 0.5 or 1.0 μM gefitinib/MTT in the absence of gefitinib) of the cells incubated in different media. (C) Effect of AREG, secreted in an autocrine manner, on the resistance of NSCLC cells to gefitinib. At the start of culture, PC-9 cells were inoculated into medium containing 1.0 μM gefitinib and recombinant AREG protein (final concentrations of 1–100 ng/ml); 72 h later, cell viability was measured by triplicate MTT assays (blue bars). The *y*-axis indicates the relative MTT values (MTT at individual concentrations of AREG/MTT without AREG) of the cells. Effect of AREG on the viability of NSCLC cells in the absence of 1.0 μM gefitinib was also studied. Individual PC-9 cells were added to medium containing recombinant AREG protein but no gefitinib; 72 h later, viability was measured by triplicate MTT assays (red bars).

biopsy samples. These five markers are cell-surface or secretory proteins and should have significant advantages for development of a novel serum maker for predicting response to gefitinib treatment, because they are presented either on the cell surface or within the extracellular space, and/or in serum, making them easily accessible as molecular markers. In fact, we were able to detect serum TGFA proteins in lung-adenocarcinoma patients by ELISA. Further evaluation of these markers for clinical use is necessary; however, the limited number of genes required for prediction should eventually enable laboratories to diagnose in advance the efficacy of gefitinib treatment for an NSCLC patient, using routine procedures such as serological examinations of blood, PCR experiments or immunohistochemical analysis of biopsy specimens.

To our knowledge, this is the first report about gene-expression profiles of unresectable 'advanced' lung cancers, although profiles of surgically resected specimens of 'early' lung cancers have been reported (17,18). However, ~70% of tumors in patients diagnosed with NCSLC are already locally advanced or metastatic, which generally renders them resistant to conventional therapeutic modalities. Therefore, the genes listed here should be useful for disclosing molecular mechanisms of lung cancer progression and may be potential targets for drug development.

Gefitinib was developed as a 'selective' inhibitor of EGFR-TK; however, no clear association between the level of EGFR activation and response to gefitinib has been found *in vitro* or *in vivo* (7,19). In clinical trials, gefitinib has been more effective against adenocarcinomas than against squamous-cell carcinomas (9,10), although over-expression of EGFR is less frequent in adenocarcinomas (20). Therefore, it is important to identify which individual tumors are good targets for this treatment. In our analysis using clinical samples, the difference in EGFR (p-EGFR)/AKT (p-AKT) protein expression and *EGFR* mutation between treatment-sensitive patients and resistant patients were not significant. On the other hand, amphiregulin (*AREG*) and transforming growth factor alpha (*TGFA*), both of which encode the ligand for EGFR and other ERBB members, were significantly over-expressed in non-responders but not (or hardly) detectable in responders ($P = 0.0000000000093$ and 0.0095, respectively; Table 4).

The results of this trial support further evaluation of the GRS system in another set of study population with NSCLC patients treated with gefitinib. The prospective trial to evaluate the reliability of several prediction systems including our GRS and controversial tests for EGFR signaling status is in progress in our institute.

The significance of the ligands and the EGFR autocrine loop in growth and survival of lung cancer cells is indisputable (20–22), but the role of AREG in formation and progression of cancers is poorly understood. However, several lines of evidence suggest that over-expression of *AREG* is associated with shortened survival of patients with NSCLC (20). Moreover, anti-apoptotic activity of AREG in human lung-adenocarcinoma cells was reported recently (21). To investigate whether the anti-apoptotic activity of AREG leads to resistance of NSCLC cells to gefitinib therapy, we performed a biological assay using a gefitinib-sensitive but *AREG*-non-expressing NSCLC cell line, PC-9. We found that the anti-tumor activity of gefitinib on PC-9 cells was dramatically decreased by autocrine secretion of AREG. This evidence strongly suggests that although growth factor signaling by the EGFR is markedly complicated at every step because of the multiplicity of ligands, dimerization partners, effectors and downstream pathways (22), AREG might be a principal activator of the ligands–receptor autocrine growth pathway that leads to cancer progression and resistance to gefitinib.

Several elements associated with the EGFR-TK pathway are present on our list of differentially expressed genes. For example, genes encoding dual specificity phosphatase 3 (*DUSP3*), *ADAM9 CD9* and *OSMR* were expressed predominantly in non-responders ($P = 0.00000000094$, 0.01, 0.000022 and 0.0000011, respectively). *DUSP3* gene modulates EGFR signaling by dephosphorylating mitogen activated protein kinase (MAPK), a key mediator of signal transduction (23), and ADAM9 is involved in activation of EGFR signaling by shedding the ectodomain of proHB-EGF (pro Heparin-binding epidermal growth factor-like growth factor) (24). CD9 physically interacts with transmembrane TGFA. CD9 expression strongly decreases the growth factor- and PMA-induced proteolytic conversions of transmembrane to soluble TGFA and strongly enhances the TGFA-induced EGFR activation (25). OSMR is reported to be constitutively associated with ERBB2 in breast cancer cells (26). Although other target molecules for gefitinib have been suggested, our results suggest that EGFR signaling containing these components is at least one of the important processes involved in response to this drug.

Since gefitinib can induce apoptosis of some cancer cells *in vivo*, other molecules with anti-apoptotic activity, as well as AREG, may contribute to a tumor's resistance to the drug. *AVEN* (apoptosis, caspase-activation inhibitor), which was specifically expressed in our non-responders ($P = 0.00000000042$), is known to enhance the anti-apoptotic activity of Bcl-xL and to suppress Apaf-1-mediated caspase activation (27). On the other hand, mechanisms regulating drug transport should also affect drug resistance. *GCLC* (glutamate–cysteine ligase, catalytic subunit), which plays an important role in cellular detoxification of anti-cancer drugs such as cisplatin, etoposide and doxorubicin (28), was over-expressed in our group of non-responders ($P = 0.00000012$). As these genes correlated negatively with responses to chemotherapy in our panel of tumors (i.e. the higher the expression of these genes, the greater the resistance to gefitinib), they might be involved in the mechanism(s) leading to that resistance. It should be noted also that the functions of nearly half of our candidate prediction genes are unknown. Therefore, further investigations will be needed to reveal more clearly the biological events underlying responses of NSCLCs to gefitinib.

CONCLUSION

We identified 51 genes whose expression differed significantly between responders and non-responders to gefitinib among human lung carcinomas, and established a numerical scoring system, based on expression patterns of 12 of those genes, to predict the response of individual tumors to this drug. Although further validation using a larger set of clinical cases will be necessary, the data presented here may yield valuable insights into the molecular events underlying signal-suppressing strategies and provide important information about gefitinib treatment for individual NSCLC patients by testing a set of genes with high predictive values.

MATERIALS AND METHODS

Patients and tissue samples

From December 2001 to November 2003, we carried out a phase II clinical study entitled 'Multi-center trial to explore the dominant biological factors responsible for clinical anti-tumor effect and pharmacokinetics of ZD1839 250 mg daily in patients with advanced non-small-cell lung cancer who have failed previous chemotherapy'. The primary endpoint was to clarify a gene-expression profile that could determine in advance a potential anti-tumor effect of gefitinib. At the beginning of the study, the rationale for the sample size was estimated from that of studies conducted thus far (29,30). Since the response rate for gefitinib was $<20\%$ in the patients of lung cancer (8–10), about 50 patients were supposed to be required to obtain learning cases estimated earlier. Patients whose locally advanced (stage IIIB) or metastasized (stage IV) NSCLCs were resistant to one or more regimens of conventional chemotherapy were enrolled in this trial. Inclusion criteria were (1) age >20 years, (2) performance status (PS) 0–2, (3) adequate liver and kidney function tests. All patients were treated with 250 mg of gefitinib orally once a day at the Tokushima University or Kinki University hospitals in Japan. The treatment was continued until the patient was dropped from the study due to (1) progression of disease, (2) intolerable toxicity, (3) withdrawal of consent.

Objective tumor responses were assessed every 4 weeks after the beginning of treatment, according to criteria outlined by the *Union International Contre le Cancer*/World Health Organization (UICC/WHO). Response categories were as follows: complete response (CR), no residual tumor in any evaluable lesion; partial response (PR), residual tumor with evidence of $\geq 50\%$ decrease under baseline in the sum of all measurable lesions, and no new lesions; progressive disease (PD), residual tumor with evidence of $\geq 25\%$ increase